Genetic architecture and behavioral analysis of attention and impulsivity

Loos, M.

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Chapter 2

Dopamine receptor D_1/D_5 gene expression in the medial prefrontal cortex predicts impulsive choice in rats

Maarten Loos, Tommy Pattij, Mieke C. W. Janssen, Danielle S. Counotte,
Anton N. M. Schoffelmeer, August B. Smit,
Sabine Spijker, Marcel M. van Gaalen

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Abstract

A neuropsychological hallmark of attention-deficit/hyperactivity disorder (ADHD) is the reduced ability to tolerate delay of reinforcement, leading to impulsive choice. Genetic association studies have implicated several genes involved in dopaminergic neurotransmission in ADHD. In this study, we investigated whether differences in the expression level of these dopamine-related genes of rats predict the individual level of impulsive choice. Among all frontostriatal brain regions tested, only in the medial prefrontal cortex (mPFC) we observed significant positive correlations between impulsive choice and transcript levels of the dopamine receptor D_1, the dopamine receptor D_5 and calcyon. Local mPFC infusions of the dopamine D_1/D_5 antagonist SCH 23390 and agonist SKF 38393 resulted in increased impulsive choice, in agreement with the idea that endogenous dopamine receptor D_1/D_5 stimulation in the mPFC promotes the choice of large delayed rewards. Together, these data indicate that this class of dopamine receptors in the mPFC plays a pivotal role in impulsive choice and aberrancies thereof might contribute to ADHD symptomatology.

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a highly disruptive, disabling disorder clinically characterized by inattention, hyperactivity and impulsiveness. ADHD patients show deficits on different neuropsychological tasks. Current theories suggest that ADHD is the result of dysfunctions in a spectrum of neuropsychological processes (Nigg *et al.*, 2005; Sergeant *et al.*, 2003; Sonuga-Barke, 2002, 2005). One of these dysfunctional processes is the inability to tolerate delay of reinforcement, as ADHD patients show increased preference for immediate small over delayed larger – and therefore more beneficial – rewards (Barkley *et al.*, 2001; Solanto *et al.*, 2001; Sonuga-Barke, 2002; Sonuga-Barke *et al.*, 1992). This results in a so-called 'impulsive choice' for the small reward. Methylphenidate (Ritalin), currently the most commonly prescribed drug for the treatment of ADHD, reduces impulsive choice in a delayed reward task in humans (Pietras *et al.*, 2003) indicating that this task relates to an important clinical symptom of ADHD.

Manipulation of dopamine, noradrenalin and serotonin neurotransmission is known to modulate impulsive choice (Robinson *et al.*, 2008; van Gaalen *et al.*, 2006b; Winstanley *et al.*, 2005; for review see Pattij & Vanderschuren, 2008). Dopaminergic neurotransmission has received particular attention in this respect, since treatment with drugs that specifically (GBR 12909) or less specifically (methylphenidate, amphetamine and cocaine) inhibit dopamine reuptake decrease impulsive choice as shown in both human (Pietras *et al.*, 2003) and rodent studies (van Gaalen *et al.*, 2006b; Wade *et al.*, 2000). Recently, genetic studies have identified associations between the occurrence of ADHD and genes involved in dopaminergic neurotransmission (for review see Waldman & Gizer, 2006),

including genes encoding the dopamine receptor D_1 (DRD1; Misener *et al.*, 2004), the dopamine receptor D_4 (DRD4; Faraone *et al.*, 2001), the dopamine receptor D_5 (DRD5; Lowe *et al.*, 2004), the dopamine transporter (SLC6A3 or DAT1; Faraone *et al.*, 2005), dopamine beta hydroxylase (DBH; Daly *et al.*, 1999) and calcyon (CALY; Laurin *et al.*, 2005). Interestingly, the latter gene encodes a protein product that is involved in regulation of the affinity state of D_1 receptors for agonists (Lidow *et al.*, 2001). Thus far, polymorphisms within the protein-coding sequence of these dopamine-related genes have not been identified, except for the 7-repeat polymorphism in the gene encoding the dopamine receptor D_4 that alters affinity to certain antipsychotics (Faraone *et al.*, 2001; Van Tol *et al.*, 1992). This may suggest that polymorphisms in genes causally related to ADHD reside in regulatory sequences instead, and exert their effect by altering the expression levels of these genes. As yet, it is unknown whether differences in gene expression of dopamine-related genes are associated with specific deficits observed in ADHD, such as impulsive choice.
Previously, we have shown that rats vary in their individual level of impulsive choice and that this phenotype is stable over time (Diergaarde *et al.*, 2008). In the present study, we investigated whether the individual level of impulsive choice could be predicted by the expression levels of the aforementioned dopamine-related genes in frontocortical and striatal brain regions, areas known to be critically involved in impulsive decision making (for reviews see Cardinal, 2006; Winstanley *et al.*, 2006a). The gene expression of two dopamine receptors (D_1 and D_5), and the protein calcyon significantly correlated with impulsive choice, notably exclusively in the infra- and prelimbic regions of the medial prefrontal cortex (mPFC). By intracranial infusions of the D_1/D_5 antagonist SCH 23390 and agonist SKF 38393 into the mPFC we examined whether D_1/D_5 receptors in the mPFC are functionally involved in impulsive choice. To rule out possible agonistic effects of SCH 23390 on 5-HT_{2c} receptors (Millan *et al.*, 2001), SCH 23390 was also co-infused with the 5-$HT_{2a/2c}$ receptor antagonist ketanserin.

Materials and Methods

Subjects

Male Wistar rats (200-250 g, Harlan CPB, Horst, The Netherlands) were housed two per cage (lights on from 7 PM to 7 AM) with water available *ad libitum* during the entire experiment. After a habituation period of 2 weeks, animals were food restricted to 85%–90% of their free feeding weight before training of the operant delayed reward task started. The Animal Care Committee of the VU University Amsterdam approved all experiments.

Delayed reward paradigm

Operant test chambers (Med Associates, St. Albans, VT) were equipped with five nose poke holes with stimulus lights and infrared detectors and a pellet dispenser (45 mg, Noyes Precision Pellets, New Brunswick, NJ) at the opposite wall. Five sessions were scheduled per week, one session each day. More details on the procedure of training the animals to perform the operant delayed reward paradigm have previously been described (van Gaalen *et al.*, 2006b). Briefly, after training for ~12 weeks, the task required animals to respond to a 10 second light stimulus in the middle hole at the beginning of each trial (initiation period), which after a response, was followed by 10 second light stimulus in the left and right hole immediately adjacent to the middle hole (choice period). A response in one hole extinguished both stimuli and delivered a small reward (one pellet) into the magazine, while a response in the other hole extinguished both stimuli and started the delay period after which a large reward (four pellets) was delivered into the magazine. For an individual rat the same hole was always associated with large reward and left and right associations were counterbalanced across rats. A session consisted of 60 trials in total, with 5 blocks of 12 trials. The first 2 trials of each block were forced trials during which once a small reward and once a large reward could be chosen (randomly presented). In the subsequent 10 trials a rat was free to choose. After each block of 12 trials, the delay period between a response into the hole associated with the large reward and delivery of the large reward was programmed to increase. No conditioned stimuli were presented during the delay period. The inter-trial interval was programmed such that the duration of each trial was 100 s. Failures to respond within the initiation period were registered as errors of omission. Rats that persistently omitted the large reward during all forced and choice trials were excluded. Percentage choice for the large reward was calculated for each block of 10 trials; for each delay [number of large rewards earned * 100 / (number of large rewards earned + number of small rewards earned)]. In this paradigm, an enhanced level of impulsive choice is defined as a reduced percentage of choice for the large reward (e.g. % impulsive choice = 100 – % choice for the large reward).

Experiment 1: Analysis of correlations between impulsive choice and the expression of dopamine-related genes

Delayed reward paradigm: After 21 training sessions a group of 24 rats learned to perform the delayed reward paradigm at the full range of delay periods (0, 10, 20, 40 and 60 s). Rats were trained at this range of delay periods for 5 additional sessions, before their preference for the large reward was monitored for 17 baseline sessions. Within each of these sessions, the individual level of impulsive choice was defined as the percentage choice for the large reward during trials with intermediate delays (10 and 20 s; a total of 20 trials). For correlation analysis, the average individual level of impulsive choice was calculated across these 17 baseline sessions.

Locomotor response to novelty: On the day between the 14th and 15th baseline session rats were transferred to a dimly lit testing room and introduced in square grey plastic boxes (50 × 50 cm) where their behavior was analyzed using a camera connected to a computer equipped with EthoVision (version 3.1, Noldus, Wageningen, The Netherlands). The total distance moved during 60 min was taken as measure of their locomotor response to novelty.
Gene expression measurements: Immediately after the 17th baseline session, rats were decapitated and brains rapidly frozen using –80 ºC isopentane. In a cryostat, the brains were sliced into 200 µm coronal sections and the following brain regions were isolated according Paxinos and Watson (1998): orbitofrontal cortex (OFC), agranular insular cortex (AI), prelimbic together with infralimbic cortex (mPFC), anterior cingulated cortex (Ac), nucleus accumbens core (NAcC) and shell (NAcS) and the medial and lateral caudate putamen (mCPU and lCPU; medial to lateral coordinates ±1.0 to ±2.0 and ±2.0 to +/-4.0 respectively). From these tissues, RNA was isolated that was DNAse-treated and reverse transcribed to cDNA using random hexanucleotide primers. Gene expression was then analyzed by real-time quantitative PCR (ABI Prism® SDS 7900, Applied Biosystems Inc., Foster City, CA, USA) using a SYBR Green approach (300 nM gene specific primers and the cDNA equivalent of 40 ng RNA in a total volume of 10 µl) and normalized to the geometric mean of 3 housekeeping genes (β-actin, Hprt and Nse). Normalized gene expression levels (GE*norm*) were calculated from detection cycle threshold values (Ct) as follows: [GE*norm* = – (Ct_{genex} – $Ct_{HKgenes}$)]. The fold difference in gene expression level between high impulsive (HI) and low impulsive (LI) groups was calculated as follows: [$2^{(GEnorm\ HI\ -\ GEnorm\ LI)}$]. In addition to the aforementioned dopamine related genes (dopamine receptors D_1 (Drd1), D_5 (Drd5), D_4 (Drd4), Dbh, Slc6a3 and Caly) also the expression of dopamine receptor D2 (Drd2s and Drd2l; the short and long variant), dopamine receptor D3 (Drd3) and the catechol-o-methyl-transferase (Comt) gene were measured. Primers were selected for a low probability of folding into loop structures, a low probability of forming primer dimers and were blasted against the NCBI rat ESTs database to check for specificity. We determined the melting curves for all primer sets used, in order to check for possible primer-dimer formation. All primers yielded a specific product of the correct melting temperature. The amplification efficiency of all primers was checked and found to be between 1.9–2.0. Nucleotide sequences of the primers are provided in Supplementary Table 1.

Experiment 2: Infusion of a dopamine D_1/D_5 receptor agonist or antagonist into the mPFC
Surgery: Before surgery, rats were trained to perform the operant delayed reward paradigm at delay periods ranging from 0 to 40 s (0, 5, 10, 20, 40) for 17 sessions. Five days before surgery operant training was stopped and food was *available ad libitum*. Rats were anaesthetized using a combination of xylazine

(Rompun; Bayer AG, Leverkusen, Germany; 7 mg/kg, i.p.) and ketamine (Alfasan; Woerden, The Netherlands; 100 mg/kg, i.m.). A double guide cannula (Plastics One, Roanoke, VA, USA) was placed above the mPFC according to coordinates derived from Paxinos and Watson (Paxinos G and C Watson 1998); anteroposterior +3.2 mm from bregma, lateral +/–0.75 mm from midline. After surgery rats were housed individually and fed *ad libitum* for one week after which they received 12 retraining sessions. Rats were ranked on the average impulsive choice of the last 3 retraining sessions, then divided into pairs, and from each pair one rat was randomly assigned to the group that would receive bilateral infralimbic infusions and the other to the group that would receive bilateral prelimbic infusions.

Drug infusions: All drugs (SCH 23390 hydrochloride (Sigma Aldrich, St. Louis, MO, USA), SKF 38393 hydrochloride (Sigma Aldrich, St. Louis, MO, USA) and ketanserin (Janssen Pharmaceutica, Beerse, Belgium) were freshly dissolved in sterile saline on the day of infusion. The drug solutions were infused through an injector that was inserted in the guide canula and protruded to the pre- or infralimbic area (3.7 or 4.9 mm below the surface of the skull). A volume of 0.5 µl was delivered at a flow rate of 0.25 µl/min using 10 µl Hamilton syringes driven by a syringe infusion pump (Harvard Apparatus, South Natick, MA, USA). Injectors were left in place for an additional 2 min to allow diffusion. Rats were placed back in their home cage and were placed in the operant chamber 10 min later. At least 2 training days separated infusion days. For all rats the order of infusions was the same; sham infusion, saline1, 0.5 µg SCH 233900, 1 µg SCH 23390, saline2, 0.05 µg SKF 38393, 0.1 µg SKF 38393, saline3, 0.1 µg ketanserin and finally 0.1 µg ketanserin followed after 10 min by 1 µg SCH 23390.

Statistical analysis

Differences between HI and LI rats were analyzed using one-way analysis of variance (ANOVA). Using the QVALUE package (Storey & Tibshirani, 2003) in the statistical software program R (version 2.7.2) the false discovery rate (FDR) was calculated for the list of p-values of the dopamine-related genes implicated in ADHD. Pearson correlations coefficients and probabilities were used to analyze correlations between impulsive choice and normalized gene expression levels. The effects of local drug infusions were analyzed using repeated-measures ANOVA, with two within-subjects factors (dose and delay) and one between-subject factor (area: infusion in either infra- or prelimbic area). If Mauchly's test for sphericity of data was significant, more conservative Greenhouse Geisser corrected probability values were reported. Where appropriate, a post hoc analysis (Fisher least square difference) was used for pair-wise comparisons of doses. All statistical analyses, except calculation of the FDR, were performed using Statistical Package for Social Sciences version 14.0 (SPSS Inc, Chicago, IL, USA).

Results

Experiment 1: Correlations between of impulsive choice and expression of dopamine-related genes

Stable individual differences in impulsive choice
The individual level of impulsive choice was calculated for all 17 baseline sessions and reliability analysis on all 23 included rats indicated that this individual level was stable across baseline sessions (Cronbach's $\alpha = 0.966$). The individual level of impulsive choice between the upper quartile (low percentage choice for large reward; HI rats) and the lower quartile (high percentage choice for large reward; LI rats) is shown in **Figure 1a**. The average impulsive choice across all baseline sessions of HI and LI rats differed significantly ($F(1,10) = 97.126$, $P < 0.0001$, **Fig. 1b**).

Impulsive choice does not correlate with errors of omission or the locomotor response to novelty
No significant differences between HI and LI rats were observed in distance moved during one hour in a novel box ($F(1,10) = 0.415$, $P = 0.534$) and in errors of omission on trials used to calculate the individual level of impulsivity ($F(1,10) = 0.530$, $P = 0.483$). Similarly, when calculated across all rats in the experiment, the individual level of impulsive choice did not correlate with errors of omission (Pearson r = 0.209, $P = 0.338$; not shown) or the distance moved (Pearson r = -0.04, $P = 0.874$; **Fig. 2a**).

Brain region-specific correlations of impulsive choice with Drd1, Drd5 and Caly
Significant differences ($P < 0.05$, FDR < 0.33) between HI and LI rats were detected in the mPFC for Drd1, Drd5 and Caly, and in the NAcS for Drd5 (**Table 1**). Correlation analysis across all 23 rats in the experiment indicated that impulsive choice correlated significantly ($P < 0.05$) with the expression of Drd1 (r = 0.52), Drd5 (r = 0.56) and Caly (r = 0.45) in the mPFC (**Fig. 2b-2d**), but not with Drd5 in the NAcS (r = 0.237, $P = 0.314$).

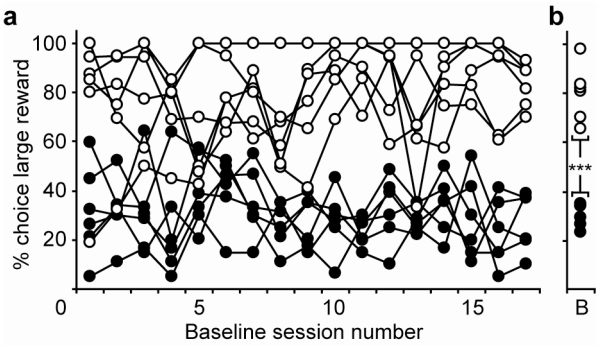

Figure 1 | Temporally stable differences in impulsive choice between rats. (**a**) The level of impulsive choice (100 – % choice large reward) of LI (white circles) and HI rats (black circles) across 17 baseline sessions. (**b**) LI and HI rats differed in their average percentage choice for large reward calculated from 17 baseline sessions (B = baseline). *** P < 0.0001.

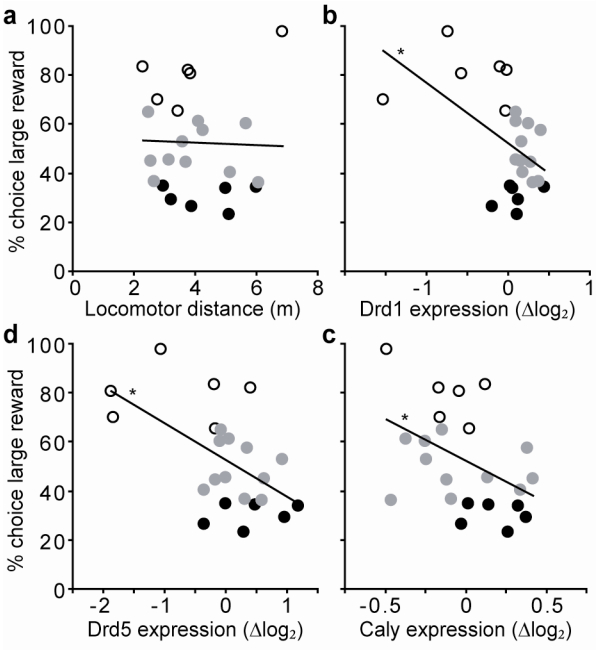

Figure 2 | Scatter plots of correlations between the level of impulsive choice (100 - % choice large reward) and (**a**) the level of locomotor activity as measured as distance moved and the level of expression in the mPFC of (**b**) Drd1, (**c**) Drd5 and (**d**) Caly. Normalized levels of gene expression are displayed as deviation from the group mean expression level (GE*norm*; log2 scale). * P < 0.05. Colors of the circles represent LI (white circles), HI rats (black circles) and intermediate impulsive rats (grey circles).

Table 1 | Differences in expression level of dopamine-related genes between HI and LI rats.

	mPFC	Ac	OFC	AI
Drd1	**1.51** (1.26–1.8)*	1.24 (1.13–1.36)	-1.03 (-1.21–0.88)	1.16 (0.88–1.53)
Drd2s	1.28 (1.08–1.51)	1.23 (1.03–1.46)	-1.39 (-1.68–1.15)	-1.05 (-1.62–0.68)
Drd2l	1.12 (0.95–1.31)	1.11 (1.02–1.21)	-1.18 (-1.36–1.03)	-1.54 (-1.99–1.19)
Drd3	na	na	na	na
Drd4	1.13 (0.99–1.29)	-1.08 (-1.49–-0.78)	1.20 (0.39–3.75)	1.24 (0.19–8.05)
Drd5	**2.32** (1.34–4.03)*	1.19 (0.88–1.61)	-1.13 (-1.38–0.92)	1.32 (1.15–1.52)
Dat1	na	na	na	na
Dbh	-1.42 (-1.77–-1.13)	-1.06 (-1.11–-1.02)	1.33 (0.72–2.48)	1.17 (0.92–1.48)
Caly	**1.23** (1.19–1.27)*	-1.07 (-1.11–-1.03)	-1.06 (-1.1–1.02)	-1.28 (-1.34–1.22)

	NAcS	NAcC	lCPU	mCPU
Drd1	1.13 (1.02–1.26)	1.03 (1.02–1.05)	1.05 (1.04–1.05)	-1.03 (-1.08–0.99)
Drd2s	1.12 (1.02–1.23)	-1.02 (-1.05–1.00)	1.00 (0.99–1.01)	1.01 (0.95–1.08)
Drd2l	1.00 (0.95–1.06)	1.05 (1.02–1.08)	1.10 (1.09–1.11)	1.01 (0.98–1.04)
Drd3	-1.03 (-1.14–0.94)	-1.01 (-1.11–0.92)	na	1.88 (0.76–4.67)
Drd4	na	na	na	na
Drd5	**1.22** (1.19–1.25)*	-1.36 (-1.63–1.14)	1.11 (1.03–1.20)	-1.11 (-1.30–0.95)
Dat1	na	na	na	-1.20 (-1.45–1.00)
Dbh	1.10 (0.89–1.37)	-1.25 (-1.54–1.01)	1.06 (0.27–4.18)	-1.00 (-1.14–0.88)
Caly	-1.12 (-1.3–0.97)	1.01 (0.99–1.04)	1.01 (0.31–3.32)	-1.02 (-1.03–1.00)

Differences are reported as fold difference where positive fold changes correspond to higher expression in HI rats. The respective 95% confidence interval of the fold difference is reported between brackets. In some brain regions reliable quantification of Dat1, Drd3 and Drd4 expression was not possible because more than 35 PCR cycles were required to reach the detection threshold, and no fold difference is reported (na). *Significant difference between HI and LI rats (**bold**).

Experiment 2: Infusion of a dopamine D_1/D_5 receptor agonist and antagonist into the mPFC

In all results described below, the between-subject factor 'area' never had a significant effect on the percentage choice for the large reward, indicating that there was no significant difference in the response to infusions between the groups receiving infralimbic and prelimbic mPFC infusions. None of the saline infusions changed the percentage choice for the large reward when compared to the next day ($saline_1$ $F(1,9) = 0.288$, $P = 0.604$; $saline_2$ $F(1,12) = 0.762$, $P = 0.400$; $saline_3$ $F(1,8) = 0.009$, $P = 0.929$). Furthermore, the percentage choice for the large reward after the first, second and third saline infusion did not differ from each other ($F(2,14) = 0.036$, $P = 0.964$). Therefore, all reported drug effects below are compared to the average percentage choice for the large reward calculated over all three saline infusions.

mPFC infusions of D_1/D_5 antagonist SCH 23390 alone and in combination with an infusion of ketanserin

The effect of mPFC infusions of two doses of the D_1/D_5 antagonist SCH 23390, as well as the effect of SCH 23390 after a preceding infusion of the serotonin 5-$HT_{2a/c}$ receptor antagonist ketanserin were statistically evaluated together (**Fig. 3a**). These infusions affected the percentage choice for the large reward significantly ($F(3,33) = 7.020$; $P < 0.001$), an effect that was not delay-dependent (dose × delay interaction $F(12,132) = 1.153$; $P = 0.324$). Post hoc analyses revealed that the percentage choice for the large reward was significantly

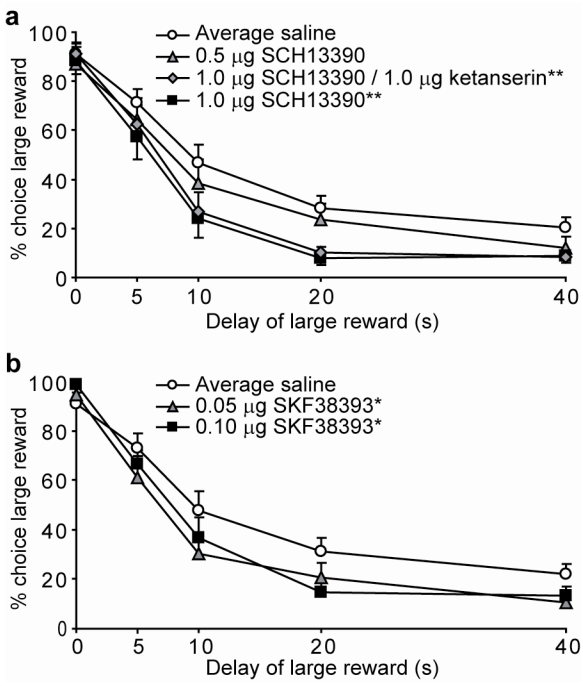

Figure 3 | Agonist and antagonist infusions of D1/D5 receptors in the mPFC increased impulsive choice. (**a**) SCH 23390 increased impulsive choice (decreased % choice large reward) compared to saline, an effect which was not blocked by co-infusion of ketanserin. (**b**) SKF 38393 increased impulsive choice compared to saline. * $P < 0.05$, ** $P < 0.01$ compared to saline. Data are expressed as mean ± SEM.

increased after an infusion of 1 μg SCH 23390 ($P < 0.001$). When an infusion of 1 μg SCH 23390 was preceded by an infusion 0.1 μg ketanserin, post hoc analyses indicated that SCH 23390 still significantly increased the percentage choice for the large reward compared to saline ($P < 0.001$). Moreover, this combination of SCH 23390 and ketanserin did not change the percentage choice for the large reward compared with a single infusion of 1 μg SCH 23390 ($P = 0.366$). Infusions of SCH 23390 alone and in combination with ketanserin also significantly increased the errors of omission (**Fig. 4**) ($F(3,33) = 3.246$; $P = 0.034$), an effect that was delay-dependent (dose × delay interaction $F(12,132) = 1.880$; $P = 0.042$). Post hoc analyses revealed that errors of omission increased significantly after 1 μg SCH 23390 when the delivery to the large reward was delayed for 20 s ($P = 0.015$) and 40 s ($P = 0.006$), an effect that was blocked by co-infusion of 0.1 μg ketanserin (**Fig. 4**).

mPFC infusions of the D_1/D_5 receptor agonist SKF 38393

Infusion of SKF 38393 increased the percentage choice for the large reward (**Fig. 3b**; $F(2,22) = 4.762$; $P = 0.019$), an effect that was not delay-dependent (dose × delay interaction $F(8,88) = 1.954$; $P = 0.062$). Post hoc analysis revealed that the percentage choice for the large reward compared to saline increased after infusion of both 0.05 μg ($P = 0.026$) and 0.1 μg ($P = 0.019$) SKF 38393. No effect of SKF 38393 on errors of omission was found ($F(2,22) = 0.239$, $P = 0.789$).

mPFC infusions of a single dose of ketanserin

There was no significant effect of infusion of 0.1 μg ketanserin on the percentage choice for the large reward ($F(1,9) = 1.307$, $P = 0.282$), its interaction with delay (dose × delay interaction $F(8,72) = 1.559$, $P = 0.153$) or errors of omission ($F(1,9) = 2.766$; $P = 0.131$).

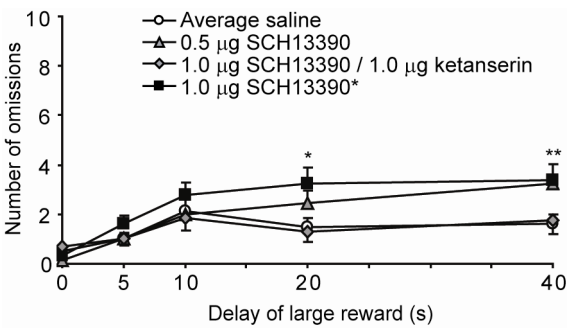

Figure 4 | Effect of drug treatment on the number of omissions. The increase in errors of omission after intra-mPFC infusion of SCH 23390 was blocked by co-infusion of ketanserin. SCH 23390 increased errors of omission (decreased % choice large reward) compared to saline, an effect which was not blocked by co-infusion of ketanserin. * P < 0.05, ** P < 0.01 compared to saline. Data are expressed as mean ± SEM.

Discussion

In this study we observed stable differences between rats in their inability to tolerate delay of reinforcement, which is a prominent neuropsychological deficit observed in ADHD (Barkley *et al.*, 2001; Solanto *et al.*, 2001; Sonuga-Barke, 2002; Sonuga-Barke *et al.*, 1992). We correlated the individual level of impulsive choice in a rodent delayed reward paradigm with the expression level of six dopamine-related genes previously associated with ADHD, and observed significant positive correlations between the expression level of three genes (Drd1, Drd5 and Caly) and impulsive choice only in the mPFC, and not in the other corticostriatal brain areas investigated. In line with previous studies (Perry *et al.*, 2005; Wilhelm & Mitchell, 2009), we found that the level of locomotor activity did not correlate with individual levels of impulsive choice. This indicates that the observed differences in impulsive choice and gene expression levels do not result from individual differences in activity. Subsequent intracranial infusions of the D_1/D_5 agonist SKF 38393 and antagonist SCH 23390 into the mPFC confirmed the role of D_1/D_5 receptor signaling in this brain area in impulsive choice.

With regard to the neuroanatomical circuitry underlying impulsive choice, different regions are thought to promote the choice of immediate small rewards and delayed large rewards (Cardinal, 2006; McClure *et al.*, 2004). A human fMRI study, demonstrated that limbic regions (including the OFC) are activated during a choice for a small immediate reward, while regions of the lateral cortex (including the dorsolateral PFC) are activated by choice irrespective of delay (McClure *et al.*, 2004). In line with this, a lesion study in rats showed that the OFC indeed promotes the choice for a small reward (Winstanley *et al.*, 2004). Lesions of the mPFC in rodents, encompassing the prelimbic region which is functionally homologous to the primate dorsolateral PFC (Uylings *et al.*, 2003), were less conclusive with respect to the role of the mPFC in impulsive choice. Rather than promoting choice for the immediate small reward, lesions to the mPFC appeared to produce some form of insensitivity to the contingencies or stimuli present in the delayed reward task (Cardinal *et al.*, 2001; Dalley *et al.*, 2004). The current findings are consistent with the aforementioned human neuroimaging data and extend previous lesioning data, as we found that modulating the dopamine D_1/D_5 tone in the mPFC increased impulsive choice. The findings with both SCH 23390 and SKF 38393 suggest that endogenous tonically- and perhaps phasically-activated dopamine D_1/D_5 receptors in the mPFC might promote the choice of large delayed rewards.

Dopamine D_1-like receptors are involved in potentiating and tuning delay-period activity of prefrontal cortical neurons (Goldman-Rakic *et al.*, 2000; Seamans & Yang, 2004) and concomitantly prevent behavioral distraction across delays between sample and retention (Robbins, 2005). The mechanisms by which mPFC D_1/D_5 receptor occupancy promotes the choice for delayed large rewards in the current study may possibly be explained by its role in maintaining a correct

representation of specific information gathered during the task. Previous studies indicated that blockade or stimulation of mPFC D_1/D_5 receptors by intracranial infusion of high doses of D_1/D_5 receptor agonists and antagonists disrupt delay period activity (Williams & Goldman-Rakic, 1995) and impair performance in delayed response tasks (Sawaguchi & Goldman-Rakic, 1991; Seamans *et al.*, 1998; Zahrt *et al.*, 1997). In the current study, infusion of the high doses of SKF 38393 and SCH 23390 may likewise have interfered with representation of task-relevant information, such as the length of the delay in a previous trial or the time that has passed with respect to the start of the session. It is conceivable that uncertain or altered representation of this information, for instance, uncertainty regarding the delay duration in the previous trial or altered session-wide timing, contributed to an accelerated discounting of the large reward.

In contrast to high doses of D_1/D_5 agents, low doses of D_1/D_5 agents have been shown to potentiate the delay-period activity of prefrontal cortical neurons (Goldman-Rakic *et al.*, 2000) and, moreover, to enhance attention in rats with low baseline performance (Granon *et al.*, 2000). Two reasons may explain why low doses of SKF 38393 and SCH 2339 did not decrease impulsive choice in the current study. First, endogenous D_1/D_5 stimulation may have been sufficient to bridge the delay times within a trial or the required session-wide temporal discrimination. In support of this argumentation, beneficial effects D_1/D_5 agonists have been observed for delays in the magnitude of 12 h, and not within the range of delays relevant for the current study, i.e. in the order of minutes and seconds (Floresco & Phillips, 2001). Second, a beneficial effect of low doses of the agonist may only have been visible in rats with a high baseline impulsive choice and presumably low dopamine D_1/D_5 receptor occupancy. Upon further categorizing rats into high, intermediate and low impulsive groups, we did not find a significant interaction between baseline impulsivity and the effect of the low doses of SKF 38393 (data not shown). However, the number of rats in this study may have been insufficient to detect small improvements, or alternatively, the employed doses of SCH 23390 and SKF 38393 were still too high to exert beneficial effects.

It should be emphasized that besides affinity for dopamine D_1/D_5 receptors, SCH 23390 may also act as an agonist of the serotonin 5-HT_{2c} receptor (Millan *et al.*, 2001). In fact, a systemic injection of the serotonin 5-$HT_{2a/2c}$ agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) has been reported to enhance impulsive choice (Evenden & Ryan, 1999). In order to disentangle D_1/D_5 and 5-HT_{2c} receptor-mediated effects, we co-infused the serotonin 5-$HT_{2a/c}$ receptor antagonist ketanserin with SCH 23390. Interestingly, the dose of ketanserin used was sufficient to attenuate the increase in omissions by SCH 23390, indicating that effects of SCH 23390 on omissions may be mediated by a non-specific effect at 5-HT_{2c} receptors. More importantly, ketanserin did not alter the increase in impulsive choice by SCH 23390, indicating that the effect of SCH 23390 on

impulsive choice is D_1/D_5 receptor-mediated and not due to its affinity for 5-HT_{2c} receptors.

Taken together, our observation that an endogenous D_1/D_5 tone in the mPFC is involved in impulsive choice is supported by the observation that dopamine release in the mPFC is enhanced during performance in the delayed reward paradigm (Winstanley *et al.*, 2006b). The current data extend this observation and suggest that the increase in dopamine during task performance may be related to the optimization of an endogenous dopamine D_1/D_5 tone to optimize or maintain a representation of task-relevant information.

In addition to the relationship between Drd1 and Drd5 gene expression in the mPFC and impulsive choice, we found a similar relationship between Caly gene expression and impulsive choice. Calcyon mRNA is abundantly expressed in regions of prefrontal cortex expressing Drd1 or Drd5 (Zelenin *et al.*, 2002) and its protein product, calycon, is involved in regulation of the affinity state of D_1 receptors for agonists (Lidow *et al.*, 2001). Although gene expression differences not necessarily predict differences in protein abundance or receptor density, collectively these data suggest the existence of a pathway through D_1/D_5 receptors and calcyon in the mPFC that is involved in impulsive choice. In fact, our observation of a positive correlation between Caly gene expression and impulsive choice is in line with a recent study showing that spontaneous hypertensive rats show both enhanced Caly gene expression (Heijtz *et al.*, 2007) and increased impulsive choice (Fox *et al.*, 2008).

The dopamine transporter has low expression in the frontal lobes (Ciliax *et al.*, 1999) and catechol-o-methyl-transferase (COMT), which degrades dopamine, has been shown to play an important role in regulating dopamine levels in this brain region (Hong *et al.*, 1998). Although COMT polymorphisms have been associated with prefrontal dopamine levels and executive functioning (for review see Tunbridge *et al.*, 2006), several studies failed to confirm the initial report (Eisenberg *et al.*, 1999) of an association between COMT and ADHD (for review see Waldman & Gizer, 2006). In the current study we also measured COMT gene expression in the mPFC, but failed to detect a significant difference between high and low impulsive rats (data not shown).

Previous genetic studies have revealed an association of human DRD1, DRD5 and CALY with ADHD. By and large, in these studies no variation in the coding region has been identified for DRD1 and CALY (Laurin *et al.*, 2005; Misener *et al.*, 2004) and, in addition, no causal polymorphism in the coding region of DRD5 has been identified thus far (Hawi *et al.*, 2003; Lowe *et al.*, 2004). These observations suggest that polymorphisms causally related to ADHD reside in regulatory sequences of these genes, thereby altering their expression levels. In this respect, two conclusions can be drawn from the current data. First, our findings clearly demonstrate that variation in the expression levels of these three aforementioned genes is correlated with impulsive choice in rats, particularly within the mPFC. Second, pharmacological manipulations of D_1/D_5 receptors

within the mPFC increase impulsive choice, further stressing the involvement of prefrontal D_1/D_5 receptors in impulsive decision making. Together, these data indicate that a pathway involving D_1/D_5 receptors and possibly calcyon in the mPFC plays a pivotal role in impulsive choice and may constitute a valuable target for further research and possible treatment of ADHD.

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Supplementary information

Supplementary Table 1 | Sequences of the primers used for real-time quantitative PCR.

	Forward	Reverse
Drd1	GGCTCCATCTCCAAGGACTGTA	AGCTTCTCCAGTGGCTTAGCTATTC
Drd2 short	CAACACCAAGCGCAGCAGT	TGGGAAACTCCCATTAGACTTCA
Drd2 long	CAACACCAAGCGCAGCAGT	GCGGGCAGCATCCTTGA
Drd3	GCCCTCTCCTCTTTGGTTTCA	GGAACGTAGAAGGACACCACTGA
Drd4	CGCCTCTGTGACACCCTCAT	CACAAACCTGTCCACGCTGAT
Drd5	GGGCCTTTCGATCACATGTCT	AAGGAAACCTCTTCCTCACAGTCA
Dat1	ATTGCATGGGCACTGCACTA	ATGGGAGGTCCATGGTGAAG
Dbh	CCCTGCGACTCCAAGATGA	CACGTGGCGACAGTAGTTGAG
Caly	GCTGCGATGACTGTGTCCAC	TCTCTCATGTCCAAAGGCTTCC
Comt	CCTGGAGGCCATCGACAC	TGCATCCATGATTTGGCCTT
β-actin	TGACCCAGATCATGTTTGAGACC	AGTGGTACGACCAGAGGCATACA
Hprt	ATGGGAGGCCATCACATTGT	ATGTAATCCAGCAGGTCAGCAA
Nse	ACGTGGTTCCATTTCAAGATGAC	CGAGCTTCAGTTAGTGCACCAA